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In vitro and *in vivo* evaluation of sanguinarine liposomes prepared by a remote loading method with three different ammonium salts

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Sanguinarine liposomes were prepared by a remote loading method using three different ammonium salts. A series of studies, including *in vitro release, in vitro* and *in vivo* anti-tumor effects and pharmacokinetics in rats, were conducted. The three liposomes showed pH-sensitive release characteristics *in vitro*, but there were obvious variations in their release profiles. Among the three liposomes, the liposomes made using ammonium citrate and phosphate possessed better anti-tumor activity *in vitro and in vivo*, compared with the liposome using ammonium sulfate. Pharmacokinetics test results in rats indicated that sanguinarine liposomes have notably elevated AUC ($P < 0.05$) and markedly lower CL ($P < 0.05$) compared with the solution, but there were no obvious differences between the three liposomes. The present study may be useful for better understanding and better choice of a suitable ammonium salt for the remote loading method.

1. Introduction

The remote loading method using ammonium salt gradient to encapsulate amphiphilic weakly basic drugs into liposomes is the most efficient way (Haran et al.1993; Bolotin et al. 1994) to improve entrapment efficiency and drug loading. Ammonium sulfate is the salt most frequently used in this method. Many drugs, including doxorubicin (Wang et al. 2000), irinotecan (Messerer et al. 2004), fasudi (Ishida et al. 2002), ciprofloxacin (Wong et al. 2003) and so on, have been encapsulated into liposomes by this method.

However, Fritze et al. (2006) have developed a novel doxorubicin liposome by the remote loading method using ammonium phosphate. Their study proved that the liposome had high encapsulation efficiency similar to liposomes prepared with ammonium sulfate. In addition, they found the liposome exhibited pH-dependent drug release which was different from the liposome prepared with ammonium sulfate.

Fritze et al. explained the difference in release profile of two liposomes, stating that as the sulfate is a salt of a stronger acid compared with phosphate, the sulfate salt will not be protonated by minor pH-shifts, e.g., from 7.4 to 5.5. In contrast, the charge density of phosphate will be decreased by protonation of the salt, resulting in the pH-sensitive release behavior. In addition, the lyotropic series favors higher solubility of doxorubicin phosphate inside the liposomes and consequently a higher release rate upon acidification.

The pH-sensitive release characteristics of liposomes prepared with ammonium phosphate might improve the applicability of liposomes to tumor sites, which exhibit a decreased pH compared with the non-tumor environment. However, no further *in vivo* effects were evaluated in their study.

Similar results were found in our studies of liposomes of an anti-tumor drug, sanguinarine (SA). Given the weakly basic nature of SA, we adopted the remote loading method to encapsulate the drug, using three different ammonium salts, ammonium sulfate, ammonium dihydrogen phosphate and ammonium citrate. These liposomes showed pH-sensitive release properties, and, furthermore, the pH release profiles of the three liposomes showed different characteristics. Based on the results, we investigated the *in vitro* and *in vivo* behavior of these liposomes.

SA is an alkaloid extracted from *Machaya microcarpa* (Wild.), a Papaveraceae plant. Many studies have shown that SA has a wide range of anti-bacterial, anti-fungal and anti-infective properties (Godowski 1989; Giuliana et al. 1997; Lopus et al. 2006), and some OTC products containing SA such as oral irrigations and toothpastes with an inhibiting effect on dental plaque and gingival infection have been developed in North America. Recent studies have shown that SA has good inhibitory activity on various cancer cells, and could reduce peripheral vascular proliferation around these cancer cells by inhibiting vascular endothelial growth factor (Eun et al. 2004). Also, it can inhibit cancer cell growth at micro-molar concentrations without affecting normal cells (Malikova et al. 2006), and it effectively block the phenomenon of Pgp-mediated MDR by inducing apoptosis through increasing the Bax/Bcl-2 ratio and activating caspase-3, and oncosis (Weerasinghe et al. 2006; Hussain et al. 2007; Serafim et al. 2008).

2. Investigations and results

2.1. Determination of liposome content

Under the defined chromatographic conditions, none of the excipients such as phospholipids and cholesterol interfere with SA determination (Fig. 1).

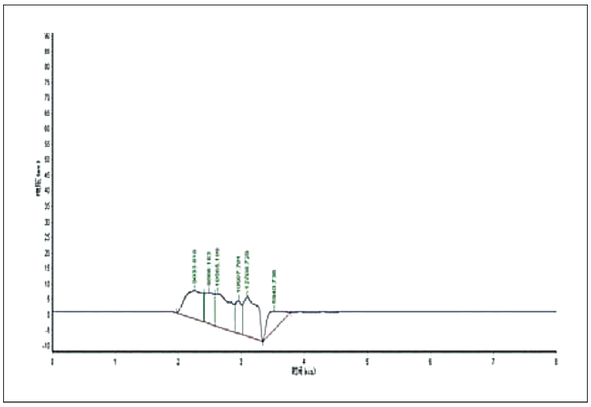
Table 1: Zeta-potential, size and polydispersity index (PI) of liposomes before and after adding SA. Data are shown as means and standard deviation (n = 3)

	Before adding SA			After adding SA		
	ξ-potential (mV)	Size (nm)	PI	ξ-potential (mV)	Size (nm)	PI
	−5.4	98.9	0.366	−10.7	101.9	0.346
SA-Lipo-S	±0.3	±0.4	±0.006	±1.0	±0.6	±0.014
	−0.7	94.4	0.303	−1.3	99.6	0.327
SA-Lipo-P	±0.2	±0.4	±0.020	±0.7	±0.2	±0.011
	−10.2	92.7	0.301	−3.3	105.5	0.323
SA-Lipo-C	±0.5	±0.5	±0.011	±1.1	±0.3	±0.015

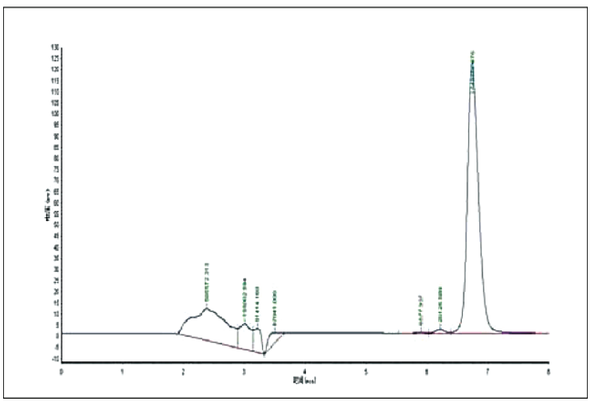
The standard curve is A = 136049C-4776.3 (n = 7), the concentration range is 0.1 ∼15 μg/mL, linear correlation coefficients (r) were more than 0.999. In addition, all the recoveries were evaluated to be 97.03–103.1%. The analytic method met the requirements for content determination.

2.2. *Physicochemical characterization of SA liposomes*

The physical properties of the three liposomes before and after adding SA are given in Table 1. The size of the liposomes before adding SA was around 100 nm. After the addition of SA, there was no significant change in the average size for any sample under investigation. The PI did not show any significant alteration, indicating that the stability of the liposomes was not adversely affected. Values for the ξ-potential were between − 1 and − 11 mV for the liposomes.



(a)



(b)

Fig. 1: HPLC chromatograms of blank liposome(A), and SA liposome (B)

The morphology of the three liposomes was different, as illustrated in Fig. 2. SA-Lipo-S showed a denser core compared with the looser interior of SA-Lipo-P and SA-Lipo-C. This may be due to the stronger acidity of sulfate, which tends to react with SA to form the un-dissociated salt with lower solubility.
The EE of SA-Lipo-S, SA-Lipo-P and SA-Lipo-C liposomes were 98.82, 98.40 and 97.53% respectively.

2.3. *In vitro drug release*

The *in vitro* release characteristics of one liposome under different pH conditions are shown in Fig. 3 and the release from different liposomes at a fixed pH is shown in Fig. 4.
The three liposomes show different pH-sensitive release characteristics *in vitro*. Among them, SA-Lipo-S presents the weakest pH-sensitivity, and the release does not vary greatly with decreased pH up to 18 h (see Fig. 3A); while the release of SA-Lipo-P and SA-Lipo-C markedly increases in an acid environment (Fig. 3B and 3C).
However, there are differences in pH-sensitivity characteristics between SA-Lipo-P and SA-Lipo-C. The release of SA-Lipo-P is relatively small at a neutral pH7.4, similar to that of SA-Lipo-S, while the release of SA-Lipo-C significantly exceeds that of

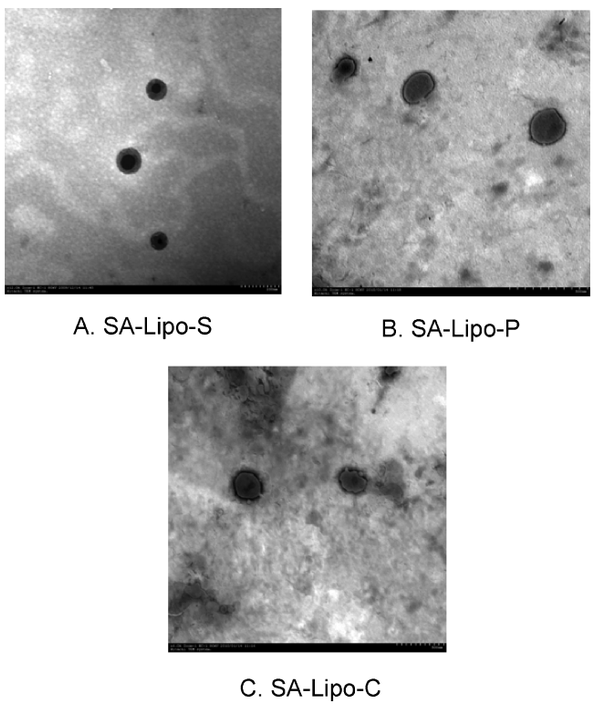
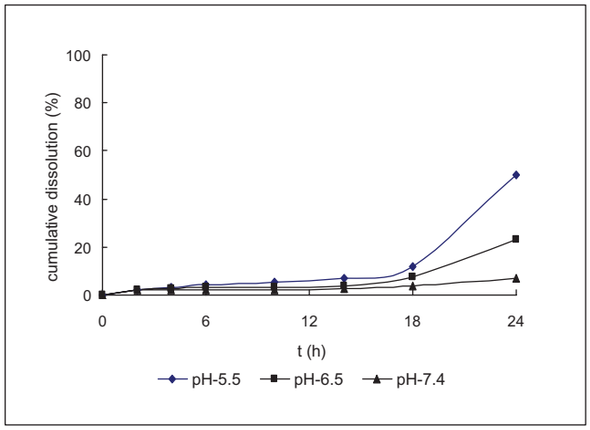
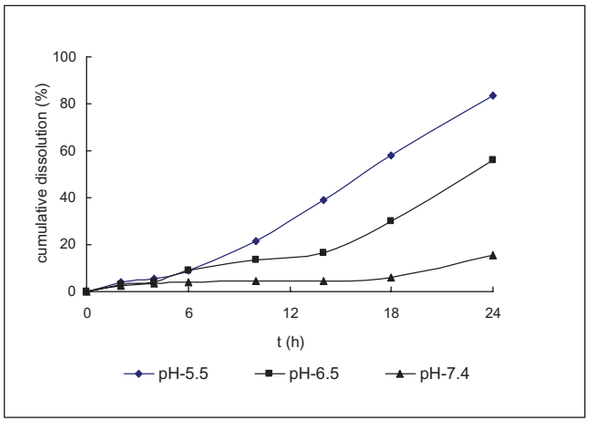


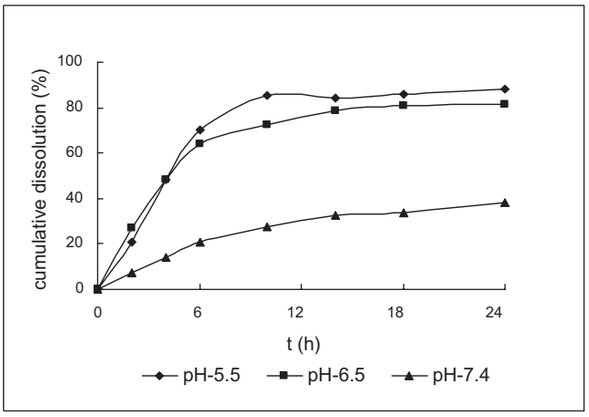
Fig. 2: Transmission electron photomicrographs of SA liposomes



A.SA-Lipo-S

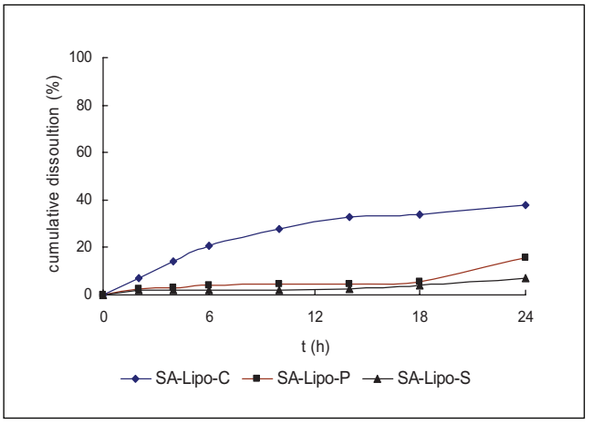


B.SA-Lipo-P

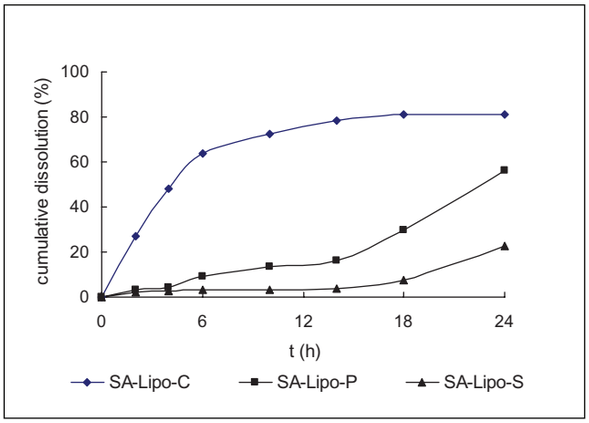


C.SA-Lipo-C

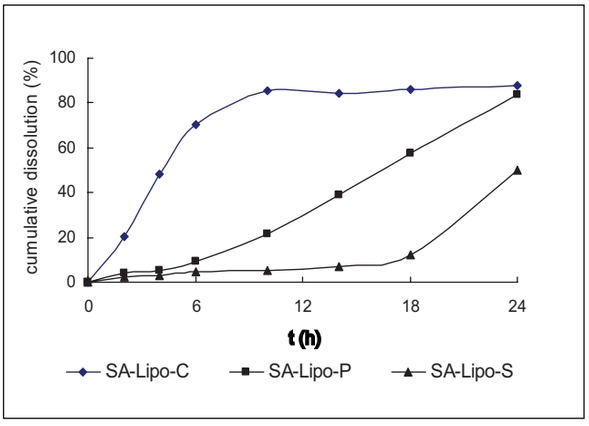
Fig. 3: Release of SA from liposomes in HEPES buffer with different pH at 37 °C



A. pH7.4



B. pH6.5



C. pH5.5

Fig. 4: Release of SA from three liposomes in HEPES buffer with fixed pH at 37 °C

SA-Lipo-P and SA-Lipo-S (Fig. 4A). At lower pH, SA-Lipo-C exhibits rapid release characteristics, the extent of release being the largest of the three liposomes under the same conditions (Fig. 4B and 4C). In contrast, the release of SA-Lipo-P is at a more moderate rate.

2.4. In vitro anti-tumor activity

The *in vitro* cytotoxic activity of various SA formulations was evaluated by the MTT assay using the HepG2 cell line. As shown in Fig. 5, the inhibition ratio of SA in the various formulations was concentration dependent, the efficiency being in the order of SA-Lipo-C > SA-Lipo-P > SA-Lipo-S > SA solution.

SA solution showed very low antitumor activity *in vitro*, compared with the liposomes. This is due to the liposomes' encapsulation of SA, which can enhance endocytosis by tumor cells; this being consistent with other published data.

Among the three liposomes, the liposome with highly pH-sensitive *in vitro* release characteristics showed better cell inhibition.

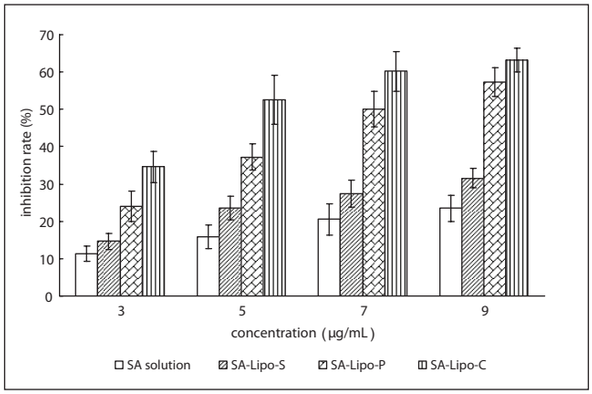


Fig. 5: Inhibition ratio of HepG2 cells with of SA solution and liposomes

2.5. In vivo tumor growth inhibition study

The *in vivo* anti-tumor efficacy of the three SA liposomes and SA solution was evaluated in Heps tumor-bearing mice. SA-Lipo-P inhibited tumor growth most efficiently, followed by SA-Lipo-C, and SA-Lipo-S, while SA solution showed the lowest inhibition rate (Table 2).

When encapsulated into liposomes, SA could reach the tumor site by passive targeting and an EPR effect (Maeda et al. 2000), thus maintaining an effective therapeutic concentration compared with the solution. So the use of liposomes to transport the drug for cancer treatment was a good choice.

2.6. Pharmacokinetic results in rats

The pharmacokinetic profiles of SA preparations are shown in Fig. 6. The data analysis of pharmacokinetic parameters was performed using Kinetica 4.4 software (China). The types of compartmental model were simulated by an open one- or two-compartment model according to Akaike's information criterion (AIC) method, respectively. The results show the two-compartment model gave the best fit for the SA solution and liposomes.

Distribution and elimination data are represented by the following parameters: the area under the plasma concentration-time curve (AUC); total body clearance (CL); the apparent volume of distribution in the central compartment (Vc); plasma half-life for the distribution and elimination phase ($t_{1/2,\alpha}$, $t_{1/2,\beta}$). All pharmacokinetic parameters evaluated are listed in Table 3.

Encapsulation of SA in liposomes produced a change in drug pharmacokinetic parameters; the liposome formulations showed an increased AUC and decreased CL compared with the solution. This may be due to more SA being delivered to the liver or spleen due to passive targeting. It therefore appears that encapsulation of SA in liposomes reduces the distribution of free drug.

Although the three liposomes showed varied pH-sensitivity release characteristics and corresponding *in vivo* or *in vitro* anti-tumor effects, there was no obvious difference between them in pharmacokinetic behavior.

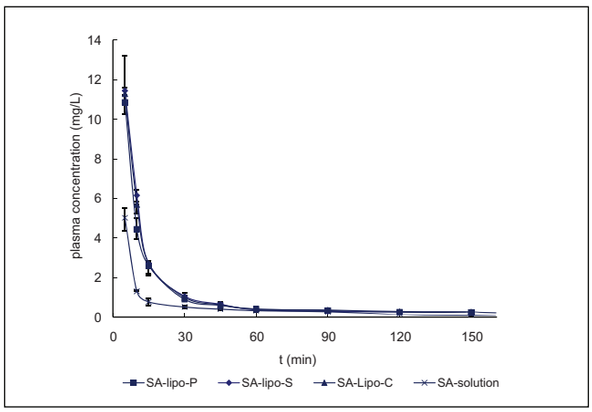


Fig. 6: Mean plasma concentration of SA vs time after intravenous administration of solution and its liposomes, separately (n = 5)

3. Discussion

Our study investigated the *in vitro* and *in vivo* effects of SA liposomes prepared using different ammonium salts, ammonium sulfate, dihydrogen phosphate and citrate.

Firstly, as in the results of Fritze et al. (2006), SA liposomes prepared by a remote loading method with three ammonium salts showed different *in vitro* pH-sensitive release behavior among them, SA-Lipo-C being the most sensitive to pH change, while SA-Lipo-S is slightly affected by the change.

The variation of pH-sensitive drug release may influence the anti-tumor efficacy of SA liposomes, as proved by *in vitro* cell viability tests. SA-Lipo-C had the best anti-tumor efficacy *in vitro*, compared with the other two liposomes. A possible cause is that after cellular internalization to endosomes with decreased pH, SA-Lipo-C, which is the most sensitive to pH change, releases more drug into the cytoplasm, thus avoiding subsequent degradation in lysosomes, and as a result better *in vitro* cell viability is obtained.

However, the results for the *in vivo* anti-tumor effect differ from the *in vitro* MTT experiment. SA-Lipo-P is superior to SA-Lipo-C, showing the best efficacy of the three liposomes. This seems to be contradictory in view of their pH-sensitive drug release behavior.

We assume the varied internal structures of liposomes made using different ammonium salts may be responsible for this phenomenon. As stated by Fritze et al., the weaker acidity of the citrate results in a lower interaction between the ion and SA and a lower ability to retain SA inside the liposomes, as can be seen from the rapid *in vitro* release profiles. Thus, more drug molecules may escape to blood circulation before the liposome reaches the tumor sites, so the anti-tumor efficacy of SA-Lipo-C *in vivo* may be less than that of SA-Lipo-P with more moderate release characteristics. Of course, the causes are very complex, and more proof is needed for further explanation.

Finally, there is no difference in pharmacokinetic parameters between the three liposomes. We assume all of them are regular liposomes, and will be rapidly eliminated from the circulation either because of leakage as a result of the interaction of plasma proteins with the liposomes or due to phagocytosis of the liposomes by the cells of the reticuloendothelial system. This can be seen from the rapid elimination phase, where differences between solution and liposomes were not significant.

Perhaps using PEG to construct a sterically stabilized liposome would be helpful to prolong circulation time and increase the AUC of SA, but several investigators have pointed out that the incorporation of PEG in lipid membranes may significantly decrease pH-dependent release from liposomes (Slepushkin VA et al. 1997), or the presence of PEG may inhibit cell uptake. So, for the explanation the difference of SA liposomes by remote loading using different ammonium salt, a regular liposome was discussed in the text, further experiments to test the possibility for sterically stabilized liposomes with pH-sensitive release property are currently in progress in our laboratories.

In conclusion, we tested the differences *in vitro* and *in vivo* between SA liposomes prepared by the remote loading method with three kinds of ammonium salts, which could result in the

Table 2: Antitumor effect of SA liposome and solution in Heps tumor-bearing mice (n = 10) $*p < 0.05$, $**p < 0.01$**, vs blank**

Group	Dose (mg/kg)	Weight of mice (g) before	after	Weight of tumor (g)	Tumor inhibition rate(%)
blank	0	21.10 ± 1.20	26.40 ± 1.71	1.57 ± 0.23	0
CTX	20	21.50 ± 0.97	22.60 ± 1.43**	0.48 ± 0.11**	69.18
SA solution	14	21.20 ± 0.92	24.30 ± 3.74	1.36 ± 0.24	13.02
SA- Lipo-S	14	21.20 ± 1.23	25.10 ± 1.37	1.12 ± 0.23**	28.33
SA-Lipo-P	14	21.40 ± 0.84	25.00 ± 1.25	0.84 ± 0.25**	46.39
SA-Lipo-C	14	21.60 ± 0.84	26.30 ± 1.70	1.05 ± 0.23**	33.12

Table 3: Pharmacokinetic parameters in rat after intravenous administration of SA solution and SA liposome (n = 5) $*p < 0.05$, $**p < 0.01$**, vs SA solution**

Parameters	SA solution	SA-Lipo-S	SA-Lipo-P	SA-Lipo-C
$T_{1/2,\alpha}$/min	2.52 ± 0.51	4.78 ± 0.41*	4.15 ± 1.60*	4.43 ± 1.40*
$T_{1/2,\beta}$/min	62.44 ± 6.44	73.65 ± 3.95*	59.08 ± 5.19	64.19 ± 8.73
CL/(L·min^{-1}·kg^{-1})	12.34 ± 3.01	8.68 ± 0.65*	9.74 ± 2.85*	8.94 ± 2.46*
AUC/(mg·min·L^{-1})	120.5 ± 43.08	182.2 ± 21.39*	168.0 ± 39.10*	175.8 ± 31.83*
$V_{(C)}$/L·kg^{-1}	71.04 ± 31.01	89.37 ± 8.91	77.02 ± 42.53	76.51 ± 25.81

variations in anti-tumor effects. As many anti-cancer drugs are weakly basic, it is usual practice to prepare liposomes by the remote loading method using ammonium salts, so the present study may help to give a better understanding of the process and to select suitable ammonium salts.

4. Experimental

4.1. Materials

SA was purchased from Nanjing Zelang Company (purity > 98%); Lipoid S100 was supplied by Lipoid GmbH (Germany); cholesterol (AR) was purchased from Shanghai Wai-hing, Biochemical Reagent Co., Ltd.; methanol and acetonitrile were of chromatographic purity. All other chemicals and reagents were of AR grade.

Calf serum was obtained from Hangzhou Sijiqing Biological Engineering Company; MTT was purchased from Fluka (USA); EDTA was from Amresco Inc.; RPMI-1640 cultural medium and DMEM cultural medium were supplied by Gibco (USA); trypan blue dye was purchased from Gibco (USA); Heps cells were supplied by Jiangsu Institute of Cancer Research; cyclophosphamide was provided by Jiangsu Hengrui Medicine Co. Ltd. (Batch No: 08061521); male Wistar rats (Grade II, n = 20, 170 g ± 20 g, Certificate No SCXK 2008-0033) were supplied by the Experimental Animal Center of Zhejiang.

4.2. Preparation of SA liposome by remote loading method

A mixture of phospholipids and cholesterol (molar ratio 2:1) was placed in a round bottomed flask and dissolved by adding the appropriate amount of dichloromethane, and a phospholipid membrane was then formed by vacuum evaporation (RE52cs rotary evaporator, Shanghai Yali-wing Biochemical Instrument Factory, China) at 30 °C to remove organic solvent. Vacuum was maintained overnight. Then 0.6 mol/L aqueous solution of ammonium salt was added to the lipid films at 30 °C to form a coarse liposome suspension. After ultrasonication (JY92 ultrasonic cell crusher, Ningbo Xinzhi Institute of Scientific Instruments, China), the liposome suspension was extruded through 0.8, 0.45 and 0.22 μm microporous membranes, respectively.

Transmembrane pH gradients were established by passing the above vesicles over a Sephadex G-50 column equilibrated with isotonic HEPES (140 mM NaCl, 10 mM HEPES, pH 7.4). Subsequently, a given concentration of SA solution was mixed with blank liposome to achieve a drug to lipid ratio of 1/7.5 (mol/mol), the loading process being carried out at 40 °C for 20 min, to obtain the SA liposome. The final drug concentration was 5.0 mM.

In accordance with the method above, three ammonium salts, ammonium sulfate, ammonium dihydrogen phosphate and ammonium citrate, were used to prepare SA liposomes, labeled SA-Lipo-S, SA-Lipo-P and SA -lipo-C, respectively.

4.3. Determination of SA content in liposomes

The HPLC system (Shimadzu LC-10AT high performance liquid chromatography system, Japan) consisted of a pump (Model LC-10A, Shimadzu, Japan), a Shim-pack CLC-ODS column (250 mm × 6 mm i.d., 5 μm, Shimadzu) maintained at 35 °C, and a UV detector (Model SPD-10A, Shimadzu, Japan) at 272 nm. The composition of the mobile phase was acetonitrile and 0.1% phosphoric acid (35:65). The mobile phase was delivered at a flow rate of 1 mL/min. The injection volume was 20 μL.

4.4. Determination of entrapment efficiency of SA liposomes

The entrapment efficiency (EE) of SA liposome was measured after separation of un-entrapped, free drug by the mini-column centrifugation method. The gel was prepared by leaving Sephadex G50 (10 g) to swell in HEPES (120 mL) at room temperature with occasional shaking for at least 5 h and was then stored at 4 °C. To prepare the mini-columns, absorbent cotton was inserted in the bottom of the barrels of 5.0 cm^3 syringes which were then filled with gel. Excess HEPES was removed by centrifugation at 1000 rpm for 3 min.

Liposome suspension (0.2 mL) was added dropwise to the center of the column, followed by centrifugation as before, and elutes containing drug-loaded liposomes were collected. The amount of drug entrapped in the liposomes was determined by HPLC after digestion in methanol. EE was calculated according to the formula: $EE = W_{liposome}/W_{total} \times 100\%$, where W_{total} is the total amount of drug in the liposome suspension and $W_{liposome}$ is the amount of drug encapsulated in the liposome.

To prove the validity of the method, 0.2 mL of saturated SA solution was applied to the mini-column instead of the liposome suspension, and after centrifugation at 1000 rpm for 3 min, no drug was detected in the elutes. This indicated that no free drug would be present when recovering the liposomes.

4.5. Physicochemical characterization of SA liposomes

The three kinds of SA liposome diluted with normal saline were morphologically characterized by transmission electron microscopy (TEM). Briefly, the diluted particles were deposited on copper grids coated with a porous polymer support. Excess sample was blotted away with filter paper. The sample was negatively stained with 2% phosphotungstic acid for 3 min. The observation and recording of images were performed with a HITACHI H-7650 transmission electron microscope (Hitachi High-Technologies Corporation Japan) at 80 kV.

The size and ξ-potential were measured using a Zetasizer 3000HS (Malvern Instruments Ltd., UK) according to the manufacturer's protocol. Dispersion Technology Software was used for analysis of effective diameter. The measurements were performed in triplicate, and the median size and range of distribution were obtained.

4.6. In vitro release of SA liposomes

To determine the drug release *in vitro*, 1.5 mL of SA liposomes were transferred into a dialysis bag with tied ends; 40 mL aliquots of 10 mM HEPES

buffer (with the addition of 140 mM NaCl) were adjusted to a series of different pH levels (7.4, 6.5 and 5.5) as the release medium. The liposomes were then incubated at 37 °C for various lengths of time. At each time point, 0.5 mL samples were removed and centrifuged at high-speed, the supernatant being analysed by HPLC to determine the drug content.

4.7. In vitro anti-tumor activity

Sensitivity of HepG2 to SA preparations was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based cytotoxicity assay (Mosmann 1983).

HepG2 cell line was purchased from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated bovine serum, 100 U/mL benzyl penicillin G and 100 ug/mL streptomycin under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C (3111 water-nested-CO_2 incubator, Thermo Electron Corporation, USA).

HepG2 cells seeded on a 96-well plate (5×10^3 cells per well) were treated with increasing concentrations of SA liposomes and SA solution, with the pure cell suspension as a blank control; the cells were cultured for 44 h and then washed twice with PBS. Then, 20 μL MTT solution (5 mg/mL in PBS) was added to each well and cells were incubated for an additional 4 h at 37 °C. The supernatant was aspirated off and 100 μL DMSO was added to the wells to dissolve any precipitate present. Optical density was measured at 570 nm using an enzyme immunoassay instrument (DJ-3200, Huadong Electron Tube Company, China), as an indicator of cell viability. Cell inhibitory ratio was calculated by the following formula: Inhibitory ratio (%) = (1- average absorbance of treated group/average absorbance of control group) × 100%.

4.8. In vivo tumor growth inhibition study

This experiment was conducted in accordance with the guideline issued by the State Food and Drug Administration (SFDA) of China. The animals were housed and cared for in accordance with the guidelines established by the National Science Council of the Republic of China.

ICR mice, weighing 18–22 g, were supplied by Shanghai SLAC Laboratory Animal Limited Company. The mice were raised in air-conditioned rooms under controlled lighting (12 h lighting/day) and were fed with standard laboratory food and water *ad libitum.*

5×10^6 Heps cells were subcutaneously inoculated into the right axillary fossa of the mice. After 24 h, the mice with implanted tumors were randomized equally into 6 groups, each group containing 10 mice. The therapy groups received the following treatment regimens: 0.9% normal saline (negative control group), 20 mg/kg cyclophosphamide (CTX, positive control group), three kinds of SA liposomes and SA solution (14 mg/kg). The treatments were given i.v. to the tail vein once a day for a total of 6 days. After the treatments, all the mice were killed and weighed simultaneously, and then the tumors were segregated and weighed.

4.9. Pharmacokinetic test in vivo

Twenty healthy Wistar rats (Grade II, n = 20, 170 g ± 20 g, Male, Certificate No SCXK 2008–0033) were divided into four groups, for the three SA liposomes and SA solution. On the day of dosing, rats received a 10 mg/kg dose of SA by tail vein injection. Doses were based on individual animal body weight.

After injection, blood samples were taken from the retro-orbital plexus at various times (0, 5, 10, 15, 30, 45, 60, 90, 120, 150, 180, 210 min), refrigerated and immediately centrifuged (5 min, 10000 × g). Plasma (100 μL) was added to 20 μL chelerythrine solution as the internal standard and 200 μL methanol. The mixture was vortexed for 3 min, followed by centrifuging at 15000 rpm for 10 min. The supernatant was analyzed by HPLC as previously described. Pharmacokinetic parameters were determined using Kinetica 4.4 software.

4.10. Statistics

Results are expressed as mean ± S.D. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. Statistical significance was evaluated by Student's *t*-test for single or multiple comparisons of experimental groups, respectively.

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